

Original Article

Knockdown of long non-coding RNA TTTY15 protects cardiomyocytes from hypoxia-induced injury by regulating let-7b/MAPK6 axis

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Abstract: Acute myocardial infarction (AMI) is a serious threat to human health. Long non-coding RNAs (lncRNAs) are known to be involved in the progression of AMI. The objective of this paper was to explore the functional effect of lncRNA testis-specific transcript Y-linked 15 (TTY15) on hypoxia-induced cardiomyocyte injury. Human cardiomyocytes AC16 were cultured under hypoxic conditions to induce cardiomyocyte injury. Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to check the expression of TTTY15, microRNA let-7b, and Mitogen-activated protein kinase 6 (MAPK6). Western blot was implemented for protein detection. Cell viability and apoptosis were examined by Cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. The target association among TTTY15, let-7b, and MAPK6 was validated by dual-luciferase reporter assay, pull-down assay and RNA immunoprecipitation (RIP) assay. We found that the abundances of TTTY15 and MAPK6 were elevated, while let-7b level declined in hypoxia-induced AC16 cells. Knockdown of TTTY15 increased cell viability, and inhibited apoptosis of hypoxia-induced AC16 cells. TTTY15 bound to and inversely regulated let-7b. Likewise, MAPK6 was a target of let-7b and was negatively regulated by let-7b. Silencing of TTTY15 ameliorated the impact of let-7b downregulation or MAPK6 upregulation on hypoxia-induced cardiomyocyte injury. TTTY15 modulated MAPK6 enrichment by sponging let-7b. In conclusion, knockdown of TTTY15 suppressed hypoxia-induced cardiomyocyte injury through the let-7b/MAPK6 axis.

Keywords: Hypoxia, human cardiomyocyte AC16, TTTY15, let-7b, MAPK6

Introduction

Acute myocardial infarction (AMI), triggered by unstable ischemic syndrome, is the main cause of cardiovascular disease-related death globally [1, 2]. The main features of AMI are inflammation, cardiomyocyte apoptosis and myocardial necrosis induced by the exigent or sustaining lack of ischemia and hypoxia [3, 4]. In the course of AMI, oxygen and nutrients supplying the myocardium would be absent due to acute blocking of the coronary artery, leading to cardiac dysfunction, even causing heart failure [2]. Emerging evidence has manifested the significance of an inflammatory response and cardiomyocyte apoptosis in AMI development, so treatment targeting inflammation and apoptosis might be valid [5].

Long non-coding RNAs (lncRNAs), a range of transcripts with length exceeding 200 nucleotides, are vital modulators in diverse biological processes of cancer biology [6]. As one type of non-coding RNAs (ncRNAs), lncRNAs have no protein-coding capacity [7]. Recently, numerous lncRNAs were validated to take part in the regulation of hypoxia-induced cardiomyocyte injury. For example, lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) knockdown intensified cardiac function in a Sprague-Dawley (SD) rat model after myocardial infarction (MI) [8]; lncRNA taurine upregulated gene1 (TUG1) exacerbated hypoxia-induced myocardial cell injury by the regulation of the miR-145-5p/Binp3 axis [9]. lncRNA testis-specific transcript Y-linked 15 (TTY15) was implicated in hypoxia-induced cardiomyocytes injury by acting as a sponge of miR-455-

TTY15 knockdown inhibits hypoxia-induced cardiomyocyte injury

5p [10]. However, the role of TTY15 still need deeper investigation.

MicroRNAs (miRNAs) are also ncRNAs, whose length is approximately 23 nucleotides [11]. They modulate gene expression post-transcriptionally through binding to the 3'-untranslated region (3'-UTR) of certain target genes [12]. Many reports confirmed that miRNAs affected myocardial hypoxia-mediated cell apoptosis. For instance, miR-429 interference weakened hypoxia-induced apoptosis through Notch1 [13]; miR-223 inhibited hypoxia-induced cell apoptosis and could moderate cell autophagy of cardiomyocytes derived from neonatal rat and H9c2 cells through targeting poly (ADP-ribose) polymerase 1 (PARP-1) [14]. MicroRNA let-7b level in plasma was recognized to be a potential indicator for AMI [15]. However, the specific role of let-7b in hypoxia-induced cardiomyocyte injury is worth of investigation.

Mitogen-activated protein kinase (MAPK6, ERK3), belonging to the MAPK subfamily, is a key member of atypical MAPK [16]. MAPK6 exerts its role by serving as a downstream gene of miRNAs, as demonstrated in cervical cancer [17], Hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC), [18] and non-small cell lung cancer (NSCLC) [19]. The role of MAPK6 in hypoxia-induced cardiomyocyte injury remained uncertain.

In this study, we built a hypoxic cardiomyocyte model using AC16 cells to explore the expression pattern and functional role of lncRNA TTY15 in hypoxia-induced AC16 cells, as well as the mechanism.

Materials and methods

Cell culture and hypoxia treatment

Human cardiomyocyte line AC16 (BNCC3377-12), purchased from BeNa culture collection (Beijing, China) was grown in Dulbecco's Modified Eagle's medium (DMEM; Solarbio, Shanghai, China) plus 10% fetal bovine serum (FBS; Solarbio), 100 U/mL penicillin (Bio Light, Shanghai, China), and 100 µg/mL streptomycin (Bio Light) in a 5% CO₂ humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and normoxic condition.

For hypoxia treatment, AC16 cells were incubated in hypoxic conditions: 5% CO₂, 5% 1% O₂ and 94% N₂ for 24 hr.

Transient transfection

Lipofectamine 2000 (Solarbio) was utilized for conducting cell transfection, as appropriate. Small interfering (si) directed against TTY15 (si-TTY15) and negative control (si-NC) were acquired from Genechem (Shanghai, China). To upregulate TTY15 or MAPK6, overexpression plasmids for pcDNA-TTY15 (TTY15) or pcDNA-MAPK6 (MAPK6) were synthesized by Hanbio Biotechnology Co., Ltd (Shanghai, China), with pcDNA (Thermo Fisher Scientific) (vector) as control. Besides, let-7b inhibitor (anti-let-7b), let-7b mimic (let-7b) and their respective controls (anti-NC and miR-NC) were provided by Generalbio (Anhui, China). Next, transfected AC16 cells were harvested after 48 hr for subsequent analyses.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Following the separation of total RNA from AC16 cells with/without transfection under normoxic/hypoxic conditions with TriQuick Reagent (Solarbio), BeyoRT™ III M-MLV reverse transcriptase (Beyotime, Shanghai, China) or miRNeasy Mini Kit (QIAGEN, Hilden, Germany) was used to generate the complementary DNA. Then, qPCR was performed with SYBR Green Realtime PCR Master Mix (Solarbio) on the ABI 7900 (Applied Biosystems, Foster City, CA, USA). The relative expression of TTY15/MAPK6 or let-7b was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6 using 2^{-ΔΔCt} cycle threshold approach. Sequences of TTY15, MAPK6, let-7b, GAPDH, and U6 primers were: TTY15, forward primer: 5'-TGAGGGAGGGATGTAGCTTTT-3', reverse primer: 5'-GAAGTCAAGCAGGCAACTGA-3'; MAPK6, forward primer: 5'-TAAAGCCATTGACATGTGGG-3', reverse primer: 5'-TCGTGCACAACAGGGATAGA-3'; let-7b, forward primer: 5'-TGAGGTAGTAGTGTGTGGTT-3', reverse primer: 5'-GCTGTCAACGATACGCTACCTA-3'; GAPDH, forward primer: 5'-CTCCTCCACCTTTGACGCTG-3', reverse primer: 5'-TCCTCTTGTGCTCTTGCTGG-3'; U6, forward primer: 5'-TGCGGGTGTCTCGCTTCGGCAGC-3', reverse primer: 5'-GTGCAGGGTCCGAGGT-3'.

Western blot

For protein level detection of MAPK6, Cleaved-caspase 3 and B-cell lymphoma-2 (Bcl-2), western blot assay was implemented. In brief, pro-

TTY15 knockdown inhibits hypoxia-induced cardiomyocyte injury

tein samples were extracted from AC16 cells with/without transfection under normoxic/hypoxic conditions by use of Radio Immunoprecipitation Assay (RIPA) buffer (Solarbio). After separation through sodium dodecyl sulphate-polyacrylamide gel electrophoresis, protein was transferred to polyvinylidene difluoride membrane (Thermo Fisher Scientific). Later, the membrane was immersed in Tris Buffered Saline + Tween containing specific primary antibodies against MAPK6 (Solarbio, K000491P, 1:1000), Cleaved-caspase 3 (Beyotime, AF12-13, 1:1000), Bcl-2 (Solarbio, K200018M, 1:1000) or β -Actin (Solarbio, K200058M, 1:5000) overnight after blockage with 5% non-fat milk. Next, the secondary antibody (Solarbio, 1:10000) was used to treat the membrane for 2 hr. Finally, the presence of signal was detected using a chemiluminescence kit (Merck Millipore, Darmstadt, Germany).

Cell counting kit-8 (CCK-8) assay

In conformity to the protocols of the manufacturer, cell viability was measured with the aid of CCK-8 solution (Beyotime). After transfection or not under normoxic/hypoxic condition, AC16 cells (approximately 5×10^3) were tiled in a 96-well plate, then 10 μ L CCK-8 solution was instilled into each well. At 4 hr post-incubation, the absorbance (at 450 nm) was tested with the help of a microplate reader (Tecan Group, Ltd., Männedorf, Switzerland).

Cell apoptosis assay

In a nutshell, AC16 cells were trypsinized, gathered, and washed with phosphate-buffered saline. Then, cells were double-stained using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit (BestBio, Shanghai, China) referring to the guidelines supplied by the manufacturer. Apoptotic cells were monitored and quantified by flow cytometer (Merck KGaA, Darmstadt, Germany).

Dual-luciferase reporter assay

The binding regions between let-7b and TTY15 or MAPK6 were predicted utilizing Starbase or TargetsCan7.2. The full fragment of TTY15 and its mutant harboring the forecasted binding-sites or mutant binding-sites were amplified by PCR and inserted into the pGL3 plasmid (Promega Corp., Madison, WI, USA) to compound TTY15-WT and TTY15-MUT, severally.

In the same way, luciferase reporter gene plasmids MAPK6-WT and MAPK6-MUT were synthesized. Then the plasmid and let-7b or miR-NC were cotransfected into AC16 cells by means of Lipofectamine 2000 (Solarbio) for 48 hr. The relative luciferase level was assessed by Dual-Luciferase reporter system (Promega Corp.).

Pull-down assay

Pull-down assay was carried out following the procedure reported previously [20]. AC16 cells were transfected with biotinylated let-7b (WT) (Bio-let-7b (WT)), Bio-let-7b (MUT) or Bio-miR-NC for 48 hr. Then, cells were lysed and incubated with streptavidin magnetic beads (Med-ChemExpress, Shanghai, China) at 4°C for 3 hr. After wash and elution, bound RNAs were subjected to the measurement of TTY15 enrichment.

RNA immunoprecipitation (RIP)

The RIP assay was conducted with the help of EZ-Magna RIP Kit (Millipore, Billerica, MA, USA). Briefly, AC16 cells were lysed by complete RIP lysis buffer then lysate supernatant was incubated with magnetic beads and human anti-Argonaute2 (ago2) antibody (Bioss, Beijing, China, bs-0297P) with human anti-IgG (Solarbio, A7001) serving as the negative control (input). After overnight-incubation, wash and proteinase K digestion, coprecipitated RNAs were subjected to analysis by qRT-PCR.

Statistical analysis

Derived from three paralleled experiments, all data were exhibited as mean \pm standard deviation (SD), which was processed by GraphPad Prism 7 (GraphPad Inc., La Jolla, CA, USA). The differences between two groups or among multiple groups were analysed by Student's *t*-test or one-way analysis of variance. Significant difference was defined as a *P*-value < 0.05.

Results

Ectopic expression of TTY15, let-7b, and MAPK6 in hypoxia-induced AC16 cells

First, qRT-PCR was employed to examine the abundance of TTY15, let-7b and MAPK6 in hypoxia-induced AC16 cells. In comparison to that in AC16 cells of control group, the expres-

TTY15 knockdown inhibits hypoxia-induced cardiomyocyte injury

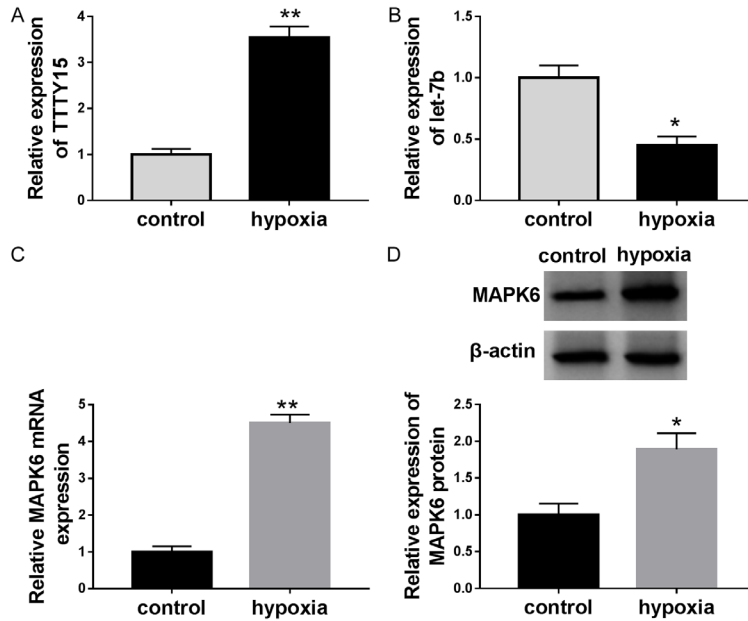


Figure 1. Ectopic expression of TTTY15, let-7b, and MAPK6 in hypoxia-induced AC16 cells. A-C. qRT-PCR was performed to determine the enrichment of TTTY15, let-7b, and MAPK6 in AC16 cells under normoxic/hypoxic conditions. D. Western blot was conducted to detect MAPK6 protein level in AC16 cells under normoxic/hypoxic conditions. * $P < 0.05$. ** $P < 0.01$.

sion levels of TTTY15 (**Figure 1A**) and MAPK6 (**Figure 1C**) were increased in hypoxia-induced AC16 cells, while let-7b expression exhibited the reverse tendency (**Figure 1B**). Besides, the protein level of MAPK6 was also higher in hypoxia-induced AC16 cells than that in cells of the control group (**Figure 1D**). Thus, we inferred that TTTY15, let-7b, and MAPK6 might play a vital part in hypoxia-induced cardiomyocyte injury.

Knockdown of TTTY15 potentiates cell viability, while inhibiting apoptosis of hypoxia-induced AC16 cells

Transfection with si-TTTY15 was implemented to interfere with TTTY15 expression, with si-NC as negative control. The following qRT-PCR validated the interference efficiency (**Figure 2A**). Then CCK-8 assay was carried out to investigate the role of TTTY15 in cell viability of hypoxia-induced AC16 cells. The data manifested that hypoxic conditions inhibited cell viability, while si-TTTY15 overturned the inhibitory effect (**Figure 2B**). Cell apoptosis assay results revealed that si-TTTY15 reversed the increase in apoptosis triggered by hypoxia (**Figure 2C, 2D**). In addition, western blot demonstrated that knockdown of TTTY15 reduced Cleaved-

caspase 3 protein level, but augmented abundance of Bcl-2 protein, suggesting that knockdown of TTTY15 constrained the apoptosis of hypoxia-induced AC16 cells (**Figure 2E-G**). To sum up, knockdown of TTTY15 hampered hypoxia-induced cardiomyocyte injury.

TTY15 serves as a sponge of let-7b and inversely regulates let-7b

Evidence suggests that lncRNAs function in multiple diseases by acting as miRNA sponges, including hypoxia-induced cardiomyocyte injury [21, 22]. Online software Starbase was utilized to predict the target miRNAs of TTTY15, and let-7b had the binding-sites (**Figure 3A**). To verify the association between TTTY15 and let-7b, dual-luciferase reporter as-

say was used. As shown in **Figure 3B**, let-7b reduced the luciferase activity of TTTY15-WT in AC16 cells, while it had no significant effect on that of TTTY15-MUT. QRT-PCR demonstrated the impact of upregulation of TTTY15 and knockdown impact of si-TTTY15 on TTTY15 expression (**Figure 3C**). Apparently, the let-7b level was repressed by TTTY15, but elevated by additional si-TTTY15 (**Figure 3D**). To further confirm that let-7b was a functional target of TTTY15, pull-down assay was performed. AC16 cells were transfected with Bio-let-7b (WT), Bio-let-7b (MUT) or Bio-miR-NC. Subsequent qRT-PCR suggested that TTTY15 was pulled down by Bio-let-7b (WT) rather than Bio-let-7b (MUT) (**Figure 3E**). Additionally, RIP disclosed that both TTTY15 and let-7b in AC16 cells were enriched in the ago2 RIP. That suggested that TTTY15 could directly interact with let-7b in AC16 cells (**Figure 3F**). Based on the above, TTTY15 functions as a sponge of let-7b.

Knockdown of TTTY15 reverses the silenced let-7b-mediated regulatory effect on hypoxia-induced cardiomyocyte injury

To further elucidate the regulatory effect of TTTY15 on let-7b, rescue experiments were executed. Hypoxia-induced AC16 cells were

TTY15 knockdown inhibits hypoxia-induced cardiomyocyte injury

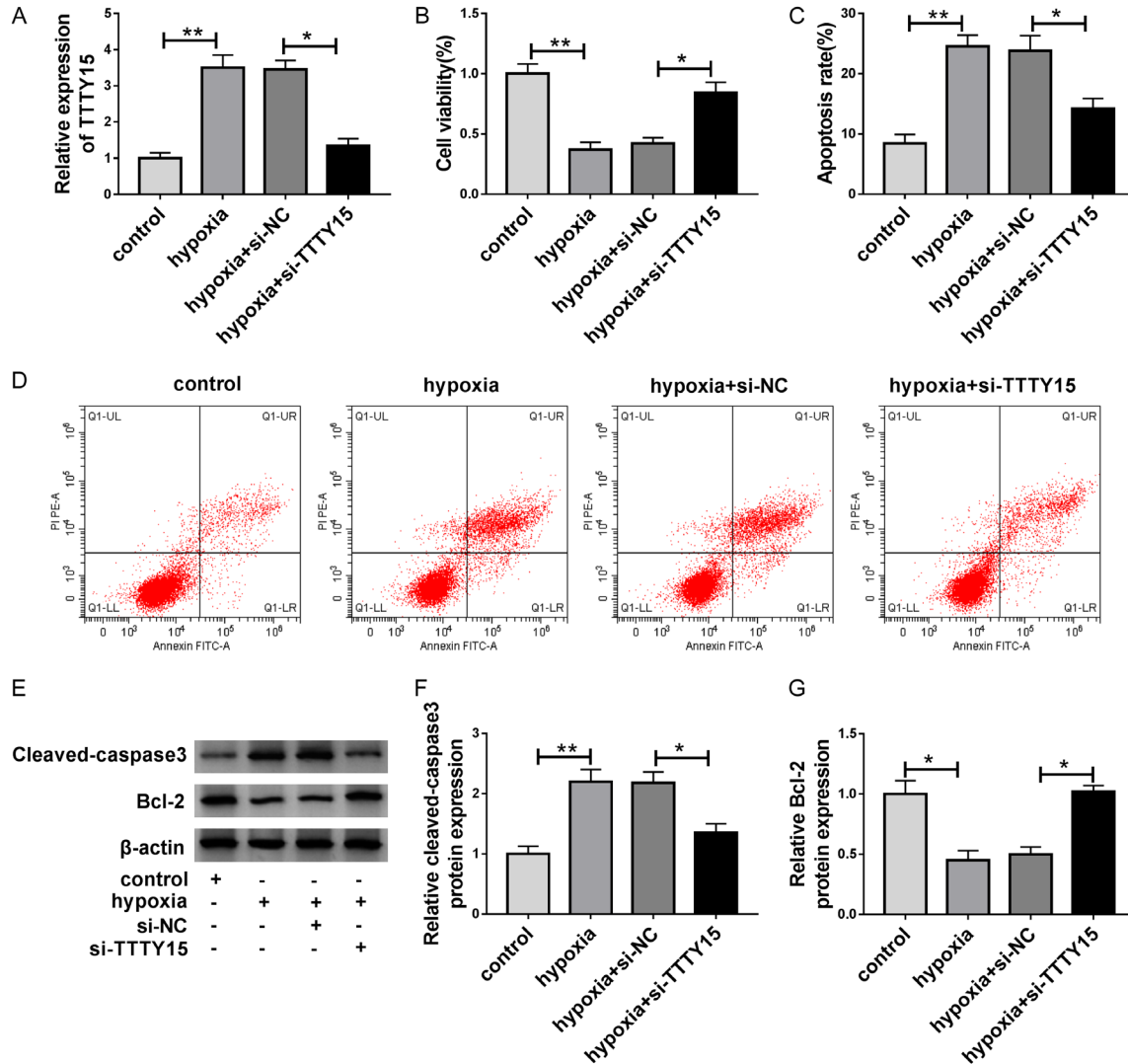


Figure 2. Knockdown of TTY15 potentiates cell viability, and inhibits apoptosis of hypoxia-induced AC16 cells. Hypoxia-induced AC16 cells were transfected with si-TTY15 or si-NC. A. qRT-PCR was applied to confirm the knockdown efficiency. B. CCK-8 assay was used for detection of cell viability. C, D. Flow cytometry was carried out to assess cell apoptosis. E-G. Western blot was used to measure the protein levels of apoptosis-related proteins Cleaved-caspase 3 and Bcl-2. * $P < 0.05$. ** $P < 0.01$.

divided into 3 groups: anti-NC + si-NC, anti-let-7b + si-NC, and anti-let-7b + si-TTY15. As exhibited in **Figure 4A**, let-7b level inhibition caused by anti-let-7b was rescued by knockdown of TTY15. With respect to the anti-let-7b + si-NC group, the anti-let-7b-induced decline in cell viability (**Figure 4B**) and elevation of apoptosis (**Figure 4C**) were both weakened by si-TTY15. Likewise, let-7b inhibitor-mediated upregulation of Cleaved caspase-3 and downregulation of Bcl-2 were both reversed in the anti-let-7b + si-TTY15 group (**Figure 4D-F**). These findings implied that TTY15 was essential for the regulatory effects of let-7b knockdown on hypoxia-induced cardiomyocyte injury.

Let-7b directly targets MAPK6

Utilizing the online TargetScan7.2 software, let-7b was predicted to specifically bind to the 3'UTR of MAPK6 (**Figure 5A**). Data from the dual-luciferase reporter assay verified that the luciferase activity of MAPK6-WT was predominantly suppressed by let-7b, while no significant change emerged in the luciferase activity of MAPK6-MUT, indicating that MAPK6 directly bound to let-7b at the predicted binding-sites (**Figure 5B**). To clarify the influence of let-7b on MAPK6 level, qRT-PCR and western blot assay were implemented and showed that let-7b significantly decreased MAPK6 expression, whereas anti-let-7b increased the MAPK6 level (Fi-

TTY15 knockdown inhibits hypoxia-induced cardiomyocyte injury

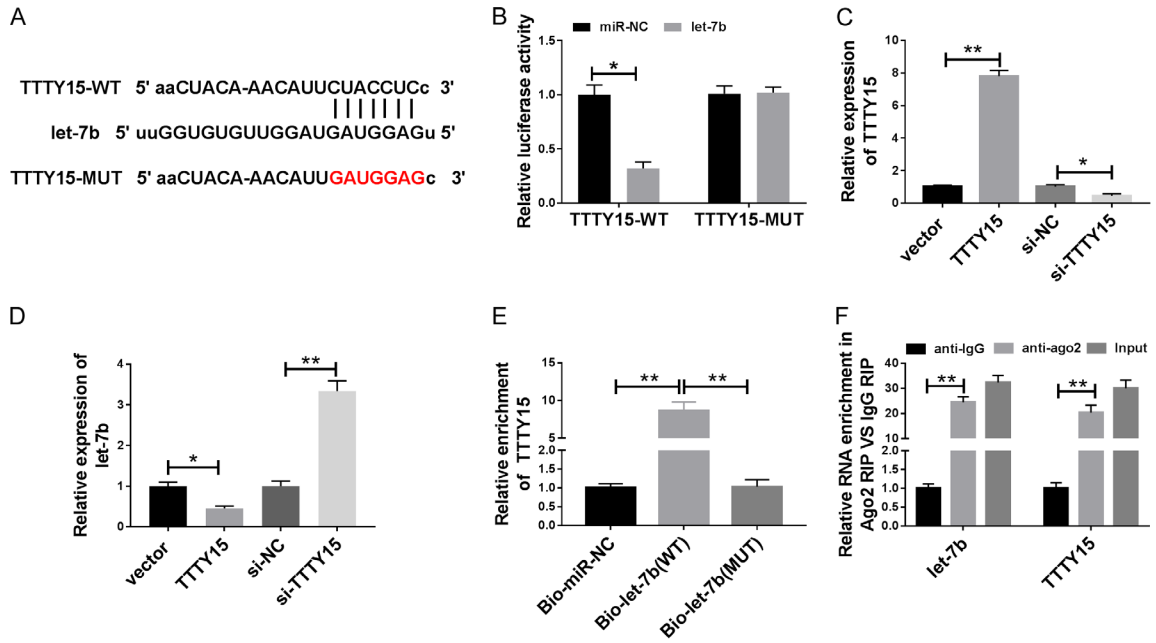


Figure 3. TTY15 serves as a sponge of let-7b and inversely regulates let-7b. **A.** The binding-sites between TTY15 and let-7b predicted by Starbase and the mutant sites are shown. **B.** Dual-luciferase reporter assay was utilized to check the luciferase activity of TTY15-WT and TTY15-MUT in AC16 cells. **C, D.** QRT-PCR was used to analyze TTY15 and let-7b expression in AC16 cells transfected with vector, TTY15, si-NC or si-TTY15. **E.** AC16 cells were transfected with Bio-miR-NC, Bio-let-7b (WT) or Bio-let-7b (MUT). Pull-down assay was executed after 48 h of transfection. TTY15 enrichment was analyzed through qRT-PCR. **F.** RIP assay was carried out. TTY15 and let-7b enrichment were detected in the samples bound to the anti-ago2 or anti-IgG in AC16 cells. * $P < 0.05$. ** $P < 0.01$.

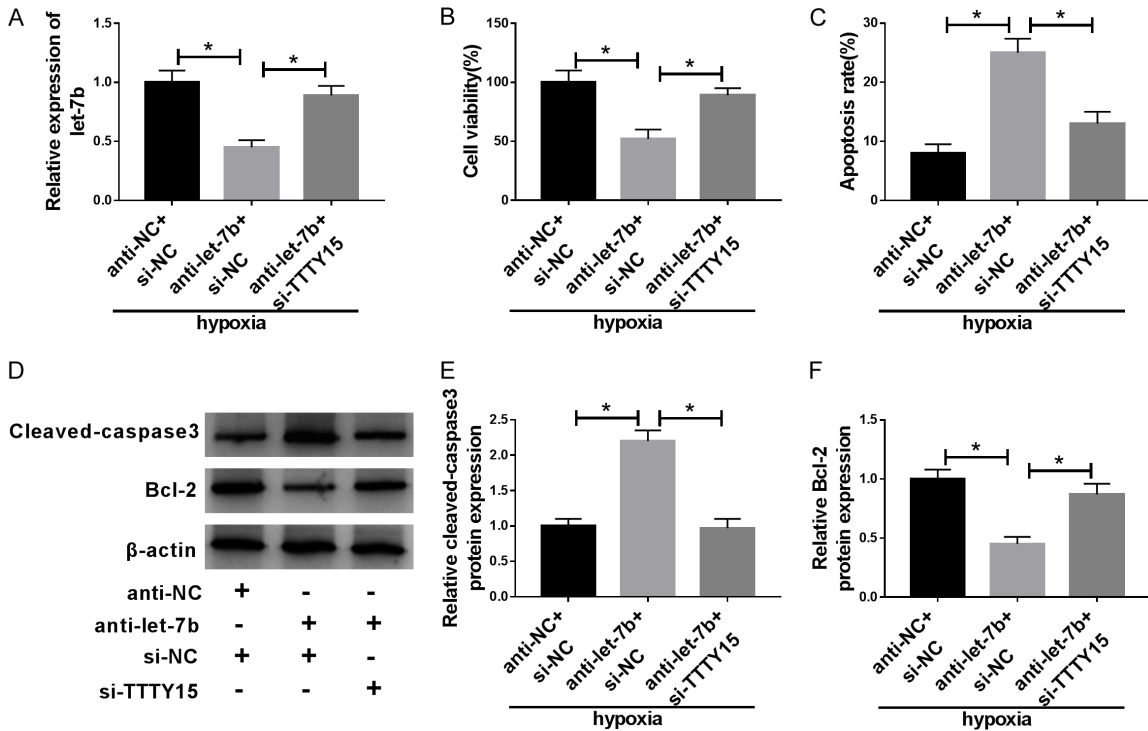


Figure 4. Knockdown of TTY15 reverses the silenced let-7b-mediated regulatory effect on hypoxia-induced cardiomyocyte injury. Hypoxia-induced AC16 cells were cotransfected with anti-NC + si-NC, anti-let-7b + si-NC, or anti-let-7b + si-TTY15. **A.** Let-7b expression in transfected hypoxia-induced AC16 cells was determined by qRT-PCR. **B.** CCK-8 assay was used to assess cell viability. **C.** Flow cytometry was done for cell apoptosis assessment. **D-F.** Western blot was carried out for evaluating Cleaved-caspase 3 and Bcl-2 protein levels. * $P < 0.05$.

TTY15 knockdown inhibits hypoxia-induced cardiomyocyte injury

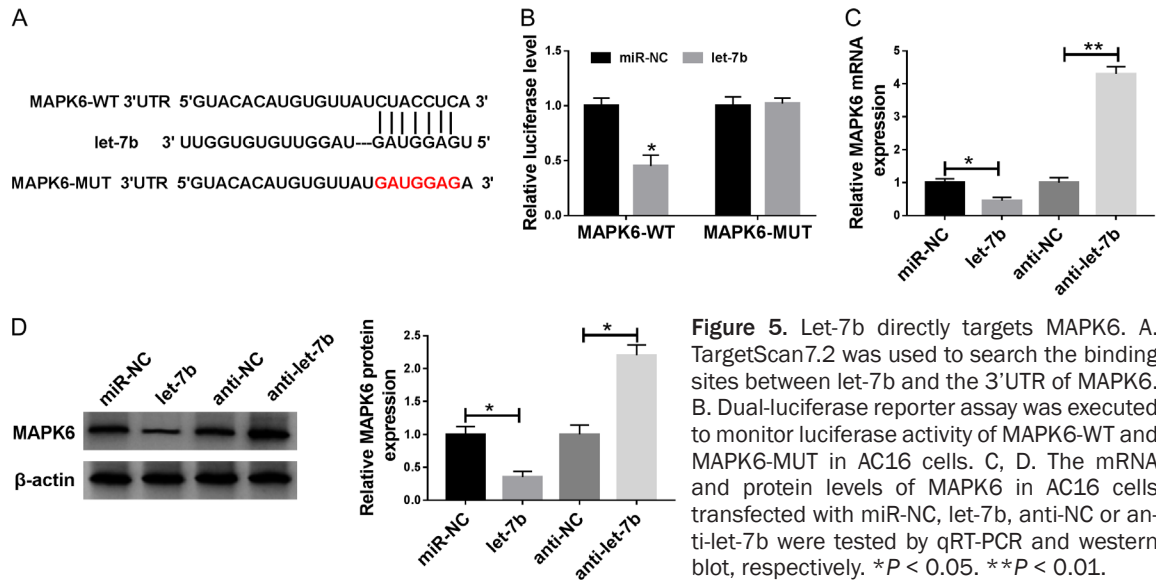


Figure 5. Let-7b directly targets MAPK6. A. TargetScan7.2 was used to search the binding sites between let-7b and the 3'UTR of MAPK6. B. Dual-luciferase reporter assay was executed to monitor luciferase activity of MAPK6-WT and MAPK6-MUT in AC16 cells. C, D. The mRNA and protein levels of MAPK6 in AC16 cells transfected with miR-NC, let-7b, anti-NC or anti-let-7b were tested by qRT-PCR and western blot, respectively. * $P < 0.05$. ** $P < 0.01$.

Figure 5C, 5D). Thus, let-7b directly targeted MAPK6 and negatively regulated its level.

Knockdown of TTTY15 also reverses the MAPK6-mediated regulatory impact on hypoxia-induced cardiomyocyte injury

Hypoxia-induced AC16 cells were cotransfected with vector + si-NC, MAPK6 + si-NC, and MAPK6 + si-TTTY15 for exploring the regulatory mechanism between TTTY15 and MAPK6 in hypoxia-induced cardiomyocyte injury. Western blot suggested that gain of MAPK6 facilitated MAPK6 protein expression, while TTTY15 knockdown abolished this promotion (Figure 6A). Moreover, MAPK6 enhancement restrained cell viability and improved apoptosis of hypoxia-induced AC16 cells, which were abrogated by introduction of si-TTTY15 (Figure 6B, 6C). Meanwhile, transfection of si-TTTY15 attenuated the increase of Cleaved caspase-3, and reduction of Bcl-2 resulted from MAPK6 upregulation (Figure 6D-F). The above findings revealed that TTTY15 was involved in MAPK6-mediated regulation of hypoxia-induced cardiomyocyte injury.

Interference against TTTY15 impedes MAPK6 expression by sponging let-7b

After transfection with si-NC, si-TTTY15, si-TTTY15 + anti-NC or si-TTTY15 + anti-let-7b, MAPK6 expression was determined by qRT-PCR and western blot assay. The results indicated that si-TTTY15 led to the reduction of MAPK6 levels, whereas let-7b inhibition eliminated this repressive effect (Figure 7A, 7B). In

other words, interference against TTTY15 hindered the MAPK6 level by elevating let-7b expression.

Discussion

With high morbidity and mortality, acute myocardial infarction (AMI) is a prevalent type of cardiovascular disease [4]. It was reported that inhibiting the apoptosis of cardiomyocytes was a key therapeutic target for AMI [23]. Here, in our study, a hypoxic cardiomyocyte model was constructed by maintaining human cardiomyocyte cell line AC16 in hypoxic conditions. First, we observed the ectopic expression of lncRNA TTTY15, microRNA let-7b, and MAPK6 in hypoxia-induced AC16 cells. Strikingly, TTTY15 and MAPK6 were upregulated, while let-7b expression was downregulated in AC16 cells under hypoxic conditions, suggesting their potential involvement in hypoxia-induced cardiomyocyte injury, which was in keeping with the preceding studies [10, 15]. Next, we evaluated the impact of hypoxia treatment on the cell viability and apoptosis of AC16 cells. By performing CCK-8, flow cytometry, and western blot assays, we proved that hypoxic treatment repressed cell viability but reinforced apoptosis of AC16 cells, indicating that a hypoxic cardiomyocyte model was established successfully.

A previous publication presented that long non-coding RNA-miRNA-mRNA axis as a molecular mechanism of cardiovascular diseases (CVDs) might help to uncover therapeutic targets, for AMI [24]. For example, Shu *et al.* claimed that

TTY15 knockdown inhibits hypoxia-induced cardiomyocyte injury

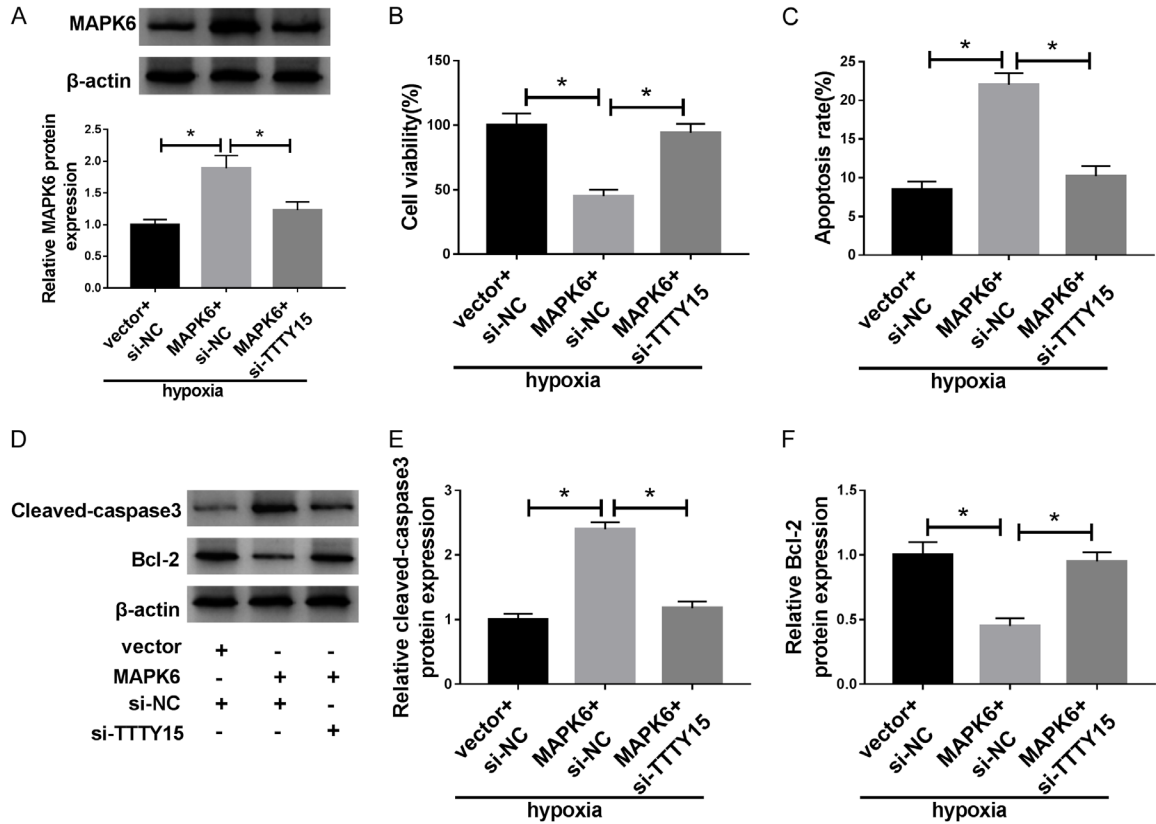


Figure 6. Knockdown of TTY15 also reverses MAPK6-mediated regulatory impact on hypoxia-induced cardiomyocyte injury. Hypoxia-induced AC16 cells were cotransfected with vector + si-NC, MAPK6 + si-NC and MAPK6 + si-TTY15. A. MAPK6 protein level was evaluated using western blot. B. CCK-8 assay was employed for cell viability detection. C. Flow cytometry was performed for evaluating cell apoptosis. D-F. A western blot was applied to measure the protein levels of Cleaved-caspase 3 and Bcl-2. * $P < 0.05$.

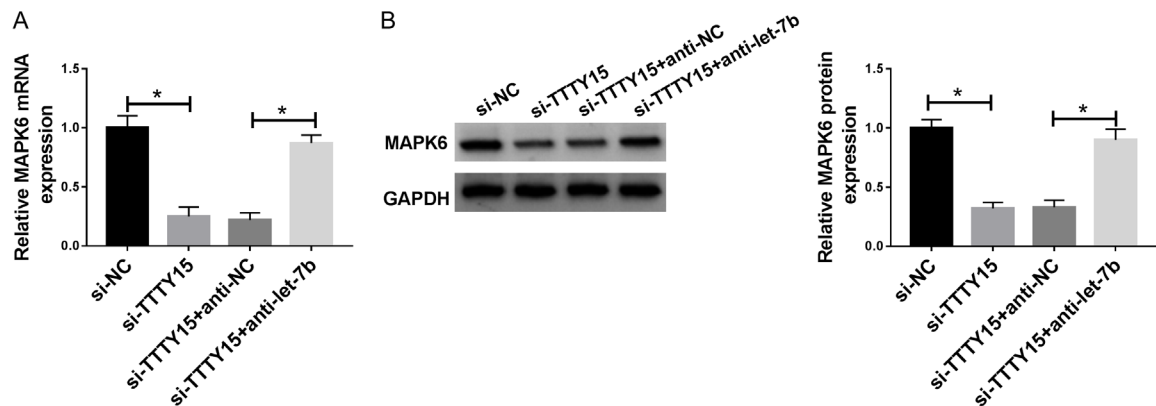


Figure 7. Interference of TTY15 impedes MAPK6 expression by sponging let-7b. AC16 cells were transfected with si-NC, si-TTY15, si-TTY15 + anti-NC or si-TTY15 + anti-let-7b. A, B. MAPK6 expression at mRNA and protein levels in transfected cells was examined through qRT-PCR and western blot, respectively. * $P < 0.05$.

silenced lncRNA antisense noncoding RNA in the INK4 locus (ANRIL) aggravated hypoxia-induced H9c2 cell injury by regulating the miR-7-5p/sirtuin 1 (SIRT1) axis [25]. LncRNA MAL-AT1 knockdown mitigated mouse AMI by the modulation of the miR-320/phosphatase and

tensin homolog deleted on chromosome 10 (Pten) axis [26]. TTY15 is located in the chromosome region Yq11.21 of male-specific regions of the Y chromosome (MSY), with 5263 bp in length [27]. TTY15 boosted prostate cancer development by binding to let-7 family

TTY15 knockdown inhibits hypoxia-induced cardiomyocyte injury

members, including let-7a, let-7c and let-7f [27]. Lai and his partners proved that TTTY15 was involved in NSCLC tumor development by T-box transcription factor 4 (TBX4) [28]. Indeed, silenced TTTY15 functioned in hypoxia-induced human cardiomyocyte primary cells (HCM) by acting as a sponge of miR-455-5p, causing apoptosis inhibition and metastasis recovery [10]. Analogously, in our study, TTTY15 knockdown contributed to cell viability, but repressed apoptosis in hypoxic cardiomyocyte model.

We then searched for the downstream miRNAs to clarify other possible mechanisms of TTTY15 in hypoxia-induced cardiomyocyte injury. Using Starbase, we discovered that let-7b could bind to TTTY15. The target relation was confirmed by dual-luciferase reporter assay, pull-down assay and RIP assay. The let-7b level was inversely regulated by TTTY15. Later, we performed rescue experiments to further explore the interaction between TTTY15 and let-7b in hypoxia-induced cardiomyocyte injury. Experimental data suggested that TTTY15 knockdown could abolish the effect of silenced let-7b on hypoxia-induced cardiomyocyte injury.

Let-7b was verified to be upregulated in serum of patients with breast cancer [29], but downregulated in malignant melanoma [30], metastatic gastric cancer [31] and AMI [15], indicating the significance of let-7b in diseases. From our data, let-7b interference reinforced hypoxia-induced cardiomyocyte injury, which was overturned by TTTY15 knockdown.

Through a bioinformatics analysis utilizing TargetScan7.2 and expression measurement, MAPK6 was identified as a target gene of let-7b, and its abundance was negatively modulated by let-7b. As a target of miR-495-3p, MAPK6 participated in NEAT1/miR-495-3p-myocardial ischemia-reperfusion injury [32]. Huang *et al.* demonstrated that MAPK6 served as an inhibitor that influenced the miR-374a-5p-induced protective impact on the hypoxia/reoxygenation H9c2 cell model [33]. The above two findings manifested that MAPK6 was involved in myocardial injury. Functional analyses for MAPK6 in our study revealed that MAPK6 had a promoting effect on hypoxia-induced cardiomyocyte injury, while this effect was undermined by TTTY15 knockdown. Additionally, MAPK6 level was co-regulated by TTTY15 and let-7b.

Taken together, we concluded that TTTY15 and MAPK6 were upregulated, but let-7b was downregulated in hypoxia-induced AC16 cells. TTTY15 positively regulated MAPK6 expression by sponging let-7b. TTTY15 knockdown kept cardiomyocytes away from hypoxia-induced injury by the let-7b/MAPK6 axis.

Disclosure of conflict of interest

None.

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TTY15 knockdown inhibits hypoxia-induced cardiomyocyte injury

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